Evidence for position effects as a variant *ETV6*-mediated leukemogenic mechanism in myeloid leukemias with a t(4;12)(q11-q12;p13) or t(5;12)(q31;p13)

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The *ETV6* gene (first identified as *TEL*) is a frequent target of chromosomal translocations in both myeloid and lymphoid leukemias. At present, more than 40 distinct translocations have been cytogenetically described, of which 13 have now also been characterized at the molecular level. These studies revealed the generation of in-frame fusion genes between different domains of ETV6 and partner genes encoding either kinases or transcription factors. However, in a number of cases—including a t(6;12)(q23;p13), the recurrent t(5;12)(q31;p13), and some cases of the t(4;12)(q11-q12;p13) described in this work—functionally significant fusions could not be identified, raising the question as to what leukemogenic mechanism is implicated in these cases. To investigate this, we have evaluated the genomic regions at 4q11-q12 and 5q31, telomeric to the breakpoints of the t(4;12)(q11-q12;p13) and t(5;12)(q31;p13). The homeobox gene *GSH2* at 4q11-q12 and the *IL-3*/*CSF2* locus at 5q31 were found to be located close to the respective breakpoints. In addition, *GSH2* and *IL-3* were found to be ectopically expressed in the leukemic cells, suggesting that expression of *GSH2* and *IL-3* was deregulated by the translocation. Our results indicate that, besides the generation of fusion transcripts, deregulation of the expression of oncogenes could be a variant leukemogenic mechanism for translocations involving the 5′ end of *ETV6,* especially for those translocations lacking functionally significant fusion transcripts. (Blood. 2002;99:1776-1784)

Introduction

Cytogenetic and molecular characterization has demonstrated that nonrandom chromosomal rearrangements in hematologic malignancies are associated with specific oncogenic mechanisms.[1,2] Translocations and inversions can result in the generation of oncogenic fusion genes or deregulated expression of protooncogenes.[1,2] The latter mechanism has been mainly described for translocations and inversions involving the immunoglobulin heavy and light chain (IgH/L) loci or T-cell receptor (TCR) loci in lymphoid leukemias and lymphomas. In these cases, expression of protooncogenes is deregulated by their juxtaposition to, respectively, B-cell– or T-cell–specific regulatory sequences of the IgH/L or TCR loci.[2] This mechanism is less common in myeloid leukemias, although some examples, such as the inv (3)(q21q26) with *RPN1*-mediated ectopic expression of *EVI1*, have been described.[3,4] The other mechanism, the generation of oncogenic fusion proteins, is observed in both lymphoid and myeloid malignancies and frequently involves the *CBFA2* (21q22), *MLL* (11q23), and *ETV6* (12p13) loci, which are known to be fused to a wide variety of partner genes.[5]

Translocations involving the *ETV6* gene are among the most intriguing aberrations found in hematologic malignancies, not only because so many different translocation partners—more than 40[5,6]—have been cytogenetically described, but also because this gene contributes to leukemogenesis through at least 3 different mechanisms. One mechanism is the modulation of the activity of the transcription factors such as CBFA2 and ARNT or the activation of the kinases PDGFRB, ABL1, ABL2, JAK2, NTRK3, and SYK by the generation of a fusion protein including the homodimerization (pointed) domain of ETV6.[7-16] A second mechanism is found in the MN1-ETV6 fusion protein that contains the DNA binding domain of ETV6[17] and was recently demonstrated to function as a chimeric transcription factor with oncogenic properties.[18] Finally, the *ETV6-MDS1/EVI1* and *ETV6-CDX2* fusions only contain the transcription/translation start of *ETV6*.[19,20] These fusions are believed to represent ectopic expression of the transcription factors *EVI1* and *CDX2*, which are not normally expressed in the hematopoietic system. These data clearly illustrate that most cloned translocations involving *ETV6* fit to the paradigm of the generation of fusion proteins, either chimeric kinases or chimeric transcription factors. However, the molecular analysis of a t(6;12)(q23;p13)[21] and the recurrent t(5;12)(q31;p13),[22] with the 5q31 breakpoints ranging from the 5′ end to the 3′ end of the *long fatty acyl-CoA synthetase 2* (*ACS2*) gene (official gene symbol *KIAA0837*), has indicated a lack of functionally significant fusion proteins, suggesting that another mechanism is involved in these translocations.

We previously described the in-frame fusion of a novel gene, *CHIC2 (BTL)*, to *ETV6* in 1 case of myeloid/natural killer (NK) cell leukemia and 3 cases of acute myeloid leukemia (AML)-M0 with a t(4;12)(q11-q12;p13).[23] Although we have characterized CHIC2 as a palmitoylated membrane-associated protein,[24] its function

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The nucleotide sequences of exon 1 and exon 2 of human *GSH2* have been submitted to GenBank with accession numbers AF306343 and AF306344.

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remains unknown and the oncogenic features of CHIC2-ETV6 are not yet understood. We describe here a further molecular analysis of the recurrent t(4;12)(q11-q12;p13) based on the characterization of 2 additional cases. Surprisingly, although the breakpoints in these cases were located within the region spanned by the P1-based artificial chromosome (PAC) contig at the *CHIC2* locus,[23] the *CHIC2-ETV6* fusion was absent and no other meaningful fusions could be detected. Based on this observation and taking into account that the recurrent t(5;12)(q31;p13) is also lacking a functionally significant fusion transcript,[22] we hypothesized that these recurrent translocations may involve the ectopic expression of protooncogenes at the partner chromosomes. To evaluate this possibility, the genomic regions telomeric to the breakpoints at 4q11-q12 and 5q31 were investigated for the presence of candidate protooncogenes, and the expression of the identified candidate genes was investigated. Our results suggest that ectopic expression of the homeobox gene *GSH2* and the growth factor gene *interleukin-3 (IL-3),* located close to the breakpoints at 4q11-q12 and 5q31, respectively, may be the underlying leukemogenic factors explaining the pathogenesis of the t(4;12)(q11-q12;p13) and t(5;12)(q31;p13).

Patients, materials, and methods

Case reports

Case 1 is a myeloid/NK cell leukemia, and cases 2, 3 and 4 are AML-M0 cases with a t(4;12)(q11-q12;p13), which were previously described.[23] Two additional cases with a t(4;12)(q11-q12;p13) were collected at the University Hospital of Navarra, Spain, (case 5) and the University Hospitals Leuven, Belgium (case 6). Case 5 (male, age 47) was diagnosed as AML-M2 and was previously described.[6] The material was obtained at relapse. Case 6 (male, age 25) presented with a de novo acute lymphocytic leukemia (ALL) at 11 years of age. He received chemotherapy over a period of 3 years. Fourteen years after the first diagnosis of ALL, he presented with therapy-related AML. Complete remission was obtained, but the patient relapsed and died of leukemia 1 year after the diagnosis of AML. Case 7 (male, age 49) was diagnosed as atypical CML (aCML) with karyotype 46,XY, t(5;12)(q31;p13) and was previously described.[25] All studies were performed on bone marrow samples, except for case 3, for which we obtained a peripheral blood sample containing less than 10% blasts.

RNA isolation and cDNA synthesis

Total RNA was isolated using Trizol (Life Technologies, Paisley, United Kingdom). Poly(A)$^+$ RNA from control bone marrow, peripheral blood, and fluorescence-activated cell sorted (FACS) cells was obtained using the mRNA isolation kit for blood/bone marrow (Roche, Mannheim, Germany). Poly(A)$^+$ RNA from fetal brain was purchased from Clontech, Palo Alto, CA. First-strand complementary DNA (cDNA) was prepared from total RNA or poly(A)$^+$ RNA using Moloney murine leukemia virus reverse transcriptase (RT) (Life Technologies) and random hexamer primers.

Rapid amplification of cDNA ends

Rapid amplification of cDNA ends (RACE) was performed as previously described.[23] For 5′-RACE on case 5, nested polymerase chain reaction (PCR) was performed using the primers ETV6R3a (5′-GAATGAGGAGATCGATAGCG-3′) and ETV6R3b (5′-TCCCTGCTCCAGTAAATTGGCTGCAAG-3′). For 3′-RACE on case 5, nested PCR was performed using the primers ETV6F1a (5′-TGAGACATGTCTGAGACTCCTGCT-3′) and ETV6F2 (5′-CCTCCAGAGAGCCCAGTGCCGAGT-3′). For 3′-RACE on case 7, nested PCR was performed using the primers ETV6F1a and ETV6F1b (5′-ACTCCTGCTCAGTGTAGCATTAAG-3′).

Reverse transcriptase–polymerase chain reaction

Standard RT-PCR was performed to assess the expression of the *CHIC2-ETV6* fusion gene and the expression of *IL-3*, *CSF2*, *IL-5*, and *IL-9*. The primers used were IL-3F (5′-AACCTGGAGGCATTCAACAG-3′) and IL-3R (5′-ACGTCAGTTTCCTCCGGAAT-3′), CSF2F (5′-AGGATGTGGCTGCAGAGC-3′) and CSF2R (5′-AGGGCAGTGCTGCTTGTAGT-3′), IL-5F (5′-CAAACGCAGAACGTTTCAGA-3′) and IL-5R (5′-CAGTACCCCCTTGCACAGTT-3′), and IL-9F (5′-CCTCTGACAACTGCACCAGA-3′) and IL-9R (5′-CTTGCCTCTCATCCCTCTCA-3′). TaqMan PCR was performed to analyze the expression of *GSH2* using the TaqMan PCR mix (Perkin Elmer, Wellesley, MA) and 10 pM of the primers GSH2TMF (5′-AGATTCCACTGCCTCACCATG-3′), GSH2TMR (5′-TCTCTCTCCAGCTCCAGGAGTT-3′), GAPDTMF (5′-AGCCTCAAGATCATCAGCAATG-3′), and GAPDTMR (5′-ATGGACTGTGGTCATGAGTCCTT-3′) together with 10 pM of the probes GSH2TMP (5′-FAM-CTCTGACGCCAGCCAGGTACCCA-TAMRA-3′) and GAPDTMP (5′-JOE-CCAACTGCTTAGCACCCCTGGCC-TAMRA-3′). The detection of *GAPD* served as internal control. The C_T values given in the text represent the number of the PCR cycle in which the fluorescent signal significantly exceeds background levels.

Generation of sequence tagged site markers

Sequence tagged sites (STSs) were designed based on available genomic sequences. The primers used were as follows: for STS-G, 5′-GCACCGCCACCACCTACAAC-3′ and 5′-GGGGATGTGAGGGAGGAGGC-3′; for STS-K, 5′-AAGCAGGAACTGTGGAAGGTG-3′ and 5′-GTGGCCAAGATAAGGGGCTA-3′; for STS-5A, 5′-TGGGGTGCACTCTGTACATC-3′ and 5′-GAACCAGTAGGCAAGGATGG-3′; for STS-3A, 5′-AAATGTTCCCCTGTGTGTGC-3′ and 5′-CCCATCATTTGTAGGCTGTTG-3′; for STS-I, IL-3F and IL-3R; and for STS-C, CSF2F and 5′-ACAGGCCCACATTCTCTCAC-3′. PCR was performed with an annealing temperature of 60°C and 30 cycles.

Constructs

The construct murine stem cell virus (MSCV)-GSH2, containing the full open reading frame of *GSH2*, was obtained by ligating a genomic fragment containing exon 1 to a PCR product containing exon 2 using the *Nco*I restriction site at the border of the 2 exons into the retroviral vector MSCV-puromycin (kindly provided by Dr D.G. Gilliland, Harvard, Boston, MA). The MSCV-GSH2delN construct, containing only the homeobox domain, and the MSCV-GSH2delH construct, containing the open reading frame of GSH2 with a deletion in the homeobox domain, were generated by PCR. The empty MSCV-puromycin vector (MSCV) served as control.

Cell culture, viral vector production, and transduction

The HEK293T and NIH3T3 cell lines were cultured in Dulbecco modified Eagle medium containing 10% fetal calf serum. NIH3T3 cells were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA). Viral vectors were produced by cotransfection of HEK293T cells with a mixture of the respective MSCV plasmids and an ecotropic packaging plasmid (Cell Genesys, Foster City, CA) using Fugene-6 (Roche). Transduction of NIH3T3 cells was performed in 6-well plates in the presence of polybrene (8 μg/mL) (Sigma, St Louis, MO). After 8 hours, the cells were washed with phosphate-buffered saline and fresh medium was added. After 24 hours, selection was started using puromycin (3 μg/mL). All experiments were started within 2 weeks after the transduction and were performed at least in double using independent transductions.

FACS of bone marrow cells

Bone marrow cells were obtained from healthy volunteers. Mononuclear cells, obtained by using a Ficoll density gradient, were separated in a CD34$^+$ and CD34$^-$ fraction by use of the magnetic-activated cell separation procedure (Miltenyi Biotec, Auburn, CA). Further fractionation was performed by incubation with the appropriate monoclonal antibodies and subsequent separation by FACS. The antibodies used were fluorescein

isothiocyanate– or phycoerythrin-conjugated anti-CD3, anti-CD7, anti-CD13, anti-CD33, anti-CD34, and anti-CD38 (Becton Dickinson, Franklin Lakes, NJ).

Genomic clones

PAC clones for the *CHIC2* locus were previously described.[23] The bacterial artificial chromosome (BAC) clone 434C1, containing more than 100 kilobases (kb) upstream and 40 kb downstream of exon 1 of *ETV6*, was identified by database searches and obtained from the RPCI11 library (Roswell Park Cancer Institute, Buffalo, NY). Other clones from the *ETV6* locus were previously described.[26,27] The PAC clones P3701 and P3704 were identified by screening of the RPCI5 library (Roswell Park Cancer Institute) using a probe derived from the 5′ end of the *ACS2* gene. The localization of cosmid c33g8 (kindly provided by Dr M. Lovett) at the *IL-3* locus was previously described.[28]

Results

Molecular cloning of additional t(4;12)(q11;p13) cases

We previously described the molecular analysis of a myeloid/NK cell leukemia and 3 AML-M0 cases with a t(4;12)(q11-q12;p13).[23] All cases were characterized by the presence of a fusion of the first 3 exons of *CHIC2* (*BTL*) to exons 2 to 8 of *ETV6* (*TEL*). For 3 of these cases, the 4q breakpoint was determined by fluorescence in situ hybridization (FISH) and mapped to the region spanned by PAC 1146G14 (Figure 1). For this study, 2 additional AML cases with a t(4;12)(q11-q12;p13) were collected. Investigation of these cases by FISH revealed the breakpoints at 12p13 to be located in intron 2 of *ETV6* for case 5 and close to exon 1 (upstream or downstream of exon 1) of *ETV6* for case 6 (Table 1). Unlike the previously reported cases, for which FISH showed split signals for PAC 1146G14, the 4q11-q12 breakpoint of case 5 was located telomeric to PAC 238H24, while the breakpoint in case 6 was located in the region spanned by PAC 200D9 and telomeric to PAC 238H24 and thus upstream of *CHIC2* (Table 1, Figure 1). This directly excluded the presence of a *CHIC2-ETV6* fusion in case 6. Attempts to amplify a *CHIC2-ETV6* fusion transcript in case 5 using primers in exon 1 of *CHIC2* and in exon 3 of *ETV6* yielded no product, whereas the control (case 1, previously described[23]) was positive, indicating that also in case 5 no *CHIC2-ETV6* fusion transcript was present (results not shown).

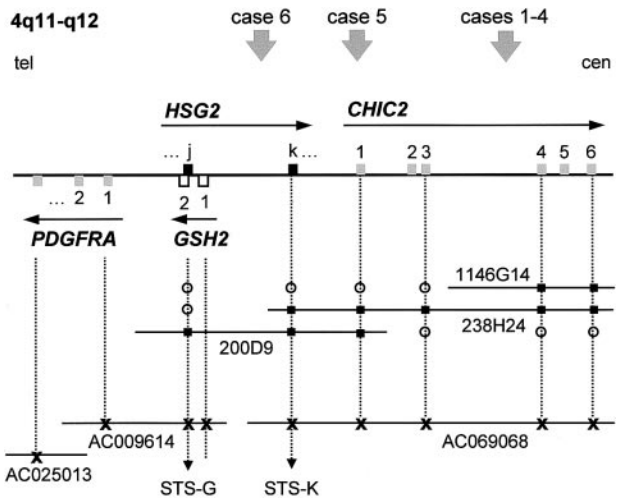

Figure 1. PAC/BAC contig covering the *CHIC2* locus at 4q11-q12 and localization of the different breakpoints of the t(4;12)(q11;p13). We previously reported the generation of a PAC contig (PACs 200D9, 238H24, and 1146G14) spanning the *CHIC2* locus at 4q11-q12.[23] Database searches identified several partially sequenced BAC clones containing the *CHIC2* and *GSH2* genes, which were aligned to the PAC contig (drawing not to scale). Using PCR and database searches, *GSH2* and the 2 exons of *HSG2* were located on this contig. The localization of the breakpoints of 6 t(4;12) cases is indicated by an arrowhead and numbered according to the cases as described in the text. Exons are represented by open or filled squares, and the transcriptional orientation is indicated by an arrow. The sequenced BAC clones are represented by their accession numbers. Tel indicates telomeric side; cen, centromeric side; open circle, negative by hybridization or PCR; filled square, positive by hybridization or PCR; x, positive by electronic PCR/database searches.

Table 1. Summary of the FISH results obtained for the 2 novel t(4;12)(q11-q12;p13) cases

Probes	(Localization)	Case 5	Case 6
4q11	*CHIC2*		
200D9	Exon 1	der(12)	split or der(12)*
238H24	Exons 1-6	split	der(4)
1146G14	Exons 4-6	der(4)	der(4)
12p13	*ETV6*		
434C1	Telomeric to exon 1/exon 1		der(4)
209J1	Exon 1/intron 1		der(12)
179A6	Exon 1/intron 1	der(4)	
50F4	Intron 1/exon 2	der(4)	
TEL11	Intron 2/exon 3	split	
54D5	Exons 5-8	der(12)	
148B6	Exon 8	der(12)	der(12)

*Only part of the metaphases showed split signals. This is indicative for a breakpoint close to the centromeric side of the region covered by PAC 200D9.

We next investigated the presence of other fusion transcripts in case 5. Using 5′-RACE starting from exon 3 of *ETV6*, transcripts were detected fusing a novel sequence to exon 2 or directly to exon 3 of *ETV6* (Figure 2). This translocation seemed to be complex, because 3′-RACE experiments showed the presence of a transcript fusing exon 2 of *ETV6* to the same sequence as identified by 5′-RACE (Figure 2), indicative of a duplication of both 12p and 4q sequences associated with the translocation. No DNA from this case was available to confirm this by Southern blotting. Unfortunately, due to lack of sufficient material, a molecular analysis could not be performed for case 6.

Cloning of human *GSH2/HSG2*

Database searches revealed 100% identity of part of the sequence obtained by 5′-RACE from case 5 to several human expressed sequence tags. These represent the *GSH2* gene; they are highly similar to the murine *Gsh2* cDNA sequence. However, the sequence fused to *ETV6* was derived from the complementary noncoding strand of *GSH2*. Part of this novel sequence overlapped with exon 2 of *GSH2* and was found to be colinear with the sequence of BAC AC009614, while the other part of the novel sequence was found to be colinear with BAC AC069068. Consensus splice donor and acceptor sequences were present at the genomic sequences, indicating that these 2 parts are 2 exons of a novel gene that is located at the *GSH2* locus but is transcribed in the opposite direction as *GSH2* and hence was named *HSG2* (Figure 1). Stop codons were found to be present in the 3 different reading frames, indicating that these exons are derived from the 5′- or 3′-untranslated region of *HSG2* or that *HSG2* is a noncoding transcript. The exact identity and complete cDNA sequence of *HSG2* was not further analyzed. Because the complete *HSG2* transcript is currently unknown, the 2 exons of *HSG2* identified in this work could not be numbered and were named exon j and exon k.

Next, the 2 exons of *GSH2* were cloned from a *Hind*III fragment of PAC 200D9. Similar to murine *Gsh2*, the complete 915 bp open

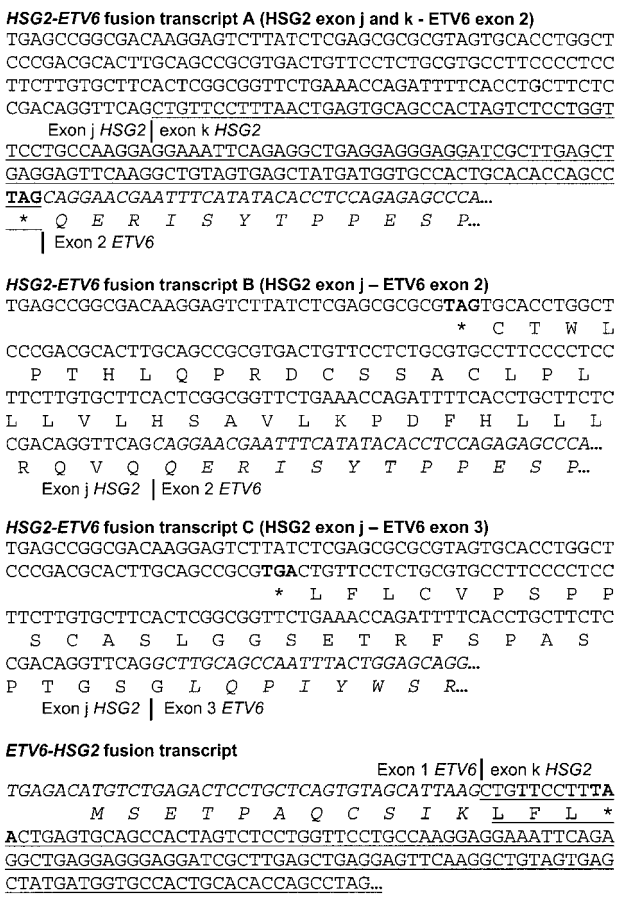

Figure 2. RACE results for case 5 indicating the different *HSG2-ETV6* and *ETV6-HSG2* fusions. The 5′- and 3′-RACE, performed on cDNA obtained from case 5, revealed the existence of 3 different *HSG2-ETV6* fusion transcripts and an *ETV6-HSG2* fusion transcript. Exon 2 of *ETV6* and exon k of *HSG2* are present in both types of fusions, which can only be explained by a duplication event associated with the translocation. Exon k of *HSG2* is underlined; sequences from *ETV6* are in italics; stop codons are in bold.

reading frame of *GSH2* was found to be present in 2 exons (sequence submitted to GenBank), separated by an intron of 2 kb. Several expressed sequence tags were found to match 100% with the exon sequences determined by us and confirmed the splice sites. In addition, RT-PCR on cDNA from human fetal brain using primers in the different exons confirmed the expression and splicing of *GSH2* (results not shown).

An STS derived for exon 2 of *GSH2* (STS-G) and for exon k of *HSG2* (STS-K), respectively, was used to assess their localization in our PAC contig spanning the *CHIC2* locus. *GSH2*, and thus also exon j of *HSG2*, was found to be present only in PAC 200D9, whereas exon k of *HSG2* was located in the region shared between the PACs 200D9 and 238H24 (Figure 1).

Taken together, these data indicated that *GSH2* was located in front of *CHIC2* and that not *GSH2*, but its antisense gene, *HSG2*, was fused to *ETV6* in case 5. Because the *HSG2* transcript contained stop codons in all reading frames, no HSG2-ETV6 fusion protein can be generated. Thus, in case 5, no functionally significant fusion transcript was generated by the translocation. However, knowing that the *GSH2* gene was in close proximity to the breakpoint in the t(4;12)(q11-q12;p13) cases and taking into account that ectopic expression of homeobox genes is a known mechanism involved in leukemia, we hypothesized that ectopic expression of *GSH2* could represent an alternative leukemogenic event. To investigate this, we analyzed the expression of *GSH2* in these cases and investigated its transforming properties using a focus formation assay in the NIH3T3 cell line.

Table 2. TaqMan real-time quantitative RT-PCR analysis of the expression of *GSH2*

Sample	C_T value*		ΔC_T
	GAPD	*GSH2*	
Fetal brain	15.5	26.0	10.5
Bone marrow	19.6 ± 0.2	Greater than 40	No expression
Peripheral blood	23.4 ± 0.2	Greater than 40	No expression
Spleen	22.6 ± 0.3	Greater than 40	No expression
HL60 cell line	16.0 ± 0.4	Greater than 40	No expression
$CD34^+$	21.1 ± 0.9	Greater than 40	No expression
$CD34^+CD38^-$	33.3 ± 0.6	Greater than 40	No expression
$CD34^+CD38^+$	24.5 ± 1.3	Greater than 40	No expression
$CD34^+CD7^+$	26.4 ± 0.2	Greater than 40	No expression
$CD34^+CD13^+CD33^+$	23.2 ± 0.5	Greater than 40	No expression
$CD34^-$	18.0 ± 1.0	Greater than 40	No expression
$CD34^-CD3^+$	27.3 ± 0.6	Greater than 40	No expression
$CD34^-CD13^+$	30.3 ± 0.6	Greater than 40	No expression
$CD34^-CD33^+$	27.7 ± 1.2	Greater than 40	No expression
BM from case 1	22.7	25.4	3.3 ± 0.8
BM from case 2	24.9	30.3	5.7 ± 0.3
PB from case 3	25.0	33.8	9.6 ± 0.7
BM from case 4	22.2	26.9	4.2 ± 0.7
BM from case 5†	28.3	37.1	9.0 ± 0.5
BM from case 6	Nd	nd	nd
Water‡	Greater than 40	Greater than 40	No expression

*The C_T value was determined in at least 3 independent experiments. It is indicated as mean ± SD when no expression of *GSH2* was detected. When expression of *GSH2* was detected, individual C_T values are given from 1 representative experiment and the mean ± SD is determined from the ΔC_T values obtained from the independent experiments.

†RNA was extracted from fixed cells.

‡Water was used as "no template control" in each experiment.

Expression of *GSH2* in the t(4;12)

We analyzed the expression of *GSH2* by use of TaqMan real-time quantitative RT-PCR using *GAPD* as an internal control. As expected, expression of *GSH2* was detected in control cDNA derived from fetal brain. No expression was detected in any of the hematopoietic tissues analyzed or in the myeloid cell line HL60. However, expression of *GSH2* was detected in the 4 cases that we previously described to contain the *CHIC2-ETV6* fusion (cases 1-4) and in the AML-M2 case (case 5), which contains the *HSG2-ETV6* fusion but lacks the *CHIC2-ETV6* fusion (Table 2). Although the C_T value for *GSH2* in case 5 was rather high, this was still different from the negative control samples. RNA from this case was extracted from fixed cells and was of poor quality, as is also clear from the higher C_T value for *GAPD*. To exclude that the expression of *GSH2* detected in these cases was the result of a clonal expansion of a $CD34^+$ cell type expressing *GSH2*, we also analyzed the expression of *GSH2* in $CD34^+$ fractions of bone marrow that was highly enriched for the respective markers (Table 1). Expression of *GSH2* could not be detected in any of these subpopulations. Both *GAPD* (Table 1) and the expression of the

Table 3. Summary of the focus formation experiment

Construct	Number of foci
MSCV-GSH2	Greater than 200
MSCV-GSH2-delN	15-35
MSCV-GSH2-delH	0-10
MSCV	0-10

homeobox gene *NKX2-3* (data not shown) were easily detected, indicating that the negative result for *GSH2* was not due to a technical problem and that the RNA extraction from these FACS-sorted cells was adequate.

Overexpression of *GSH2* transforms NIH3T3 cells

To investigate the transforming properties of *GSH2* in vitro, we retrovirally transduced the NIH3T3 cell line with the empty MSCV vector or with the MSCV vector containing wild-type *GSH2* (MSCV-GSH2), *GSH2* with a deletion of the N-terminus (MSCV-GSH2delN), or *GSH2* with a deletion of the homeobox domain (MSCV-GSH2delH), respectively. Focus formation was determined as the ability to induce a significant larger number of foci than observed after transduction of the empty vector. Experiments with the different constructs were performed at the same time, with the same batch of cells. The number of spontaneous foci observed for the empty vector was 10 at maximum. In contrast, overexpression of wild-type *GSH2* resulted in the presence of more than 200 small foci per 10 cm dish (Figure 3A,B, Table 2). The deletion mutants of *GSH2* induced only a small number of foci, comparable to the empty vector (Table 3). Typically, these foci were larger in size than the foci induced by overexpression of *GSH2* (Figure 3D), although microscopically the foci induced by *GSH2* were clearly composed of transformed cells but apparently grew more slowly (Figure 3GH). We also tested whether expression of the *CHIC2-ETV6* fusion (MSCV-CHIC2ETV6) had transforming properties, but expression of *CHIC2-ETV6* did not induce focus formation in the NIH3T3 cell line (results not shown).

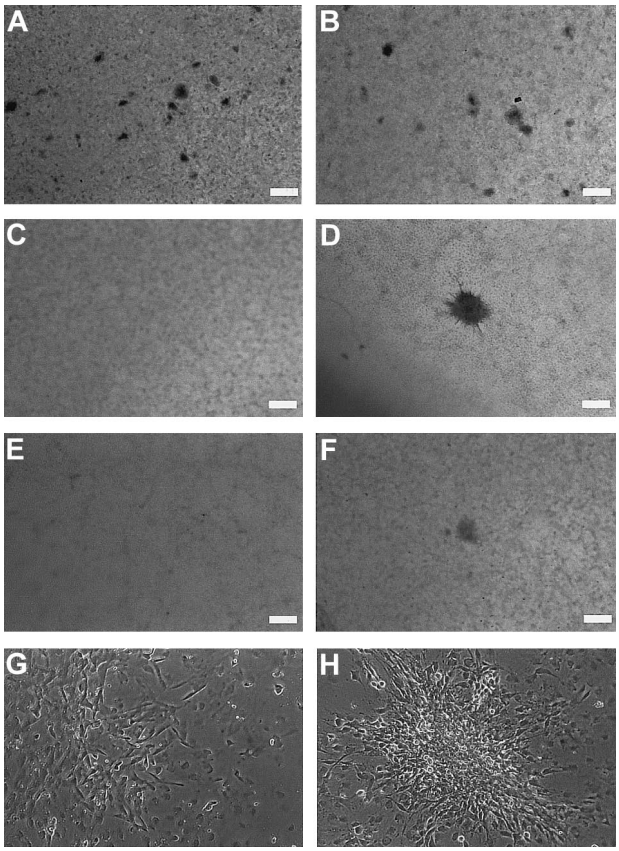

Figure 3. Focus formation assay illustrating the oncogenic potential of *GSH2*. Monolayer of cells overexpressing wild-type *GSH2* (A,B: 2 different frames of the same dish), indicating a high number (> 200 foci/10 cm dish) of relatively small foci, compared with the monolayer of cells transduced with the empty MSCV vector (C). In the latter case, spontanous foci (0-10 foci/10 cm dish) were observed. Typically, these foci were relatively larger than the foci observed with *GSH2* overexpression (compare panels A and B with D). The N-terminal deletion mutant and homeobox deletion mutant of *GSH2* induced only a low number of foci, similar to the empty vector. The monolayers observed with these construct are shown in panels E and F. (A-F: scale bar = 2 mm). The morphology and size of the foci observed for these constructs were similar to the foci observed for the empty vector. Although the cells overexpressing *GSH2* generated relatively small foci, higher-magnification pictures (G,H) clearly show the presence of transformed cells growing on top of the monolayer.

Molecular analysis of an atypical CML case with a t(5;12)(q31;p13)

The t(5;12)(q31;p13) has been described as a recurrent translocation occurring in various myeloid malignancies (at least 18 cases were previously described), often associated with eosinophilia.[22,25,29-32] We collected an atypical CML (aCML) case with a t(5;12)(q31;p13) as the sole chromosomal abnormality and investigated the presence of a possible fusion transcript. This case was described previously, and the breakpoint in *ETV6* was mapped to intron 1.[25] RACE experiments revealed the presence of an *ETV6-ACS2* fusion, confirming that this case was similar to the cases reported by Yagasaki et al.[22] Further analysis by RT-PCR revealed the presence of 3 different *ETV6-ACS2* fusion transcripts, among which was an in-frame fusion fusing the first 11 amino acids of ETV6 to almost the complete ACS2 protein (Figure 4). According to the RT-PCR results, the breakpoint in *ACS2* was at the 5′ end of this gene, similar to case 3 described by Yagasaki and coworkers.[22]

However, in the 3 cases studied by Yagasaki and colleagues, only out-of-frame *ETV6-ACS2* fusion transcripts were identified, showing that the t(5;12)(q31;p13) does not generate a common in-frame fusion gene. The exact leukemogenic factor responsible for the pathogenesis of the t(5;12)(q31;p13) thus remained unresolved. According to these observations, the only invariant result of this translocation is the juxtaposition of the region directly telomeric to *ACS2* with the *ETV6* locus. We therefore investigated whether this could lead to the juxtaposition of a protooncogene on 5q31 to *ETV6* and if this could better explain the leukemogenic mechanism of this translocation.

Analysis of the region telomeric to the breakpoint at 5q31 revealed the presence of the *IL-3/CSF2* locus in the genomic sequence of BAC CTD-2198K16 (accession no. AC034228) that also contained the 5′ end of *ACS2*. This was confirmed by further analysis of this region using the available genomic sequences (accession numbers are listed in Figure 5) and by mapping of 2 PAC clones (P3701 and P3704) and a cosmid (c33g8) by PCR using STS markers specific for the 5′ end of *ACS2* (STS-5A) and the 3′ end of *ACS2* (STS-3A), *IL-3* (STS-I), and *CSF2* (STS-C) (Figure 5). A complete 1 Mb contig encompassing the *ACS2*, *IL-3*, *CSF2*, *IRF1*, *IL-5*, and *IL-4* loci was constructed previously,[33] although the exact orientation of this contig remained unclear. This was due mainly to the small physical distance between the *IL-3* and *IL-5* loci, which made FISH-based mapping difficult and resulted in contradictions in the literature.[28,34] The cloning of 4 t(5;12) cases with an *ETV6-ACS2* fusion and the known telomere to centromere orientation of *ETV6*[26] are strongly suggestive for a telomere to centromere orientation of *ACS2*, enabling orientation of the contig described by Frazer et al[33] as centromere–*ACS2*–*IL3*– *CSF2*–*IRF1*–*IL5*–*IL4*–telomere. In agreement with this, Yagasaki et al[22] mapped the *IRF1* gene telomeric to the 5q31 breakpoint by FISH, and Grimaldi et al[35] cloned the t(5;14)(q31;q32) breakpoint, also confirming that *CSF2* is located telomeric to *IL-3*. Taken together, all these data are consistent with a localization of *IL-3* immediately telomeric to *ACS2*, as shown in Figure 5. Because *ACS2* and *IL-3* were both present in PAC P3704 and according to the contig

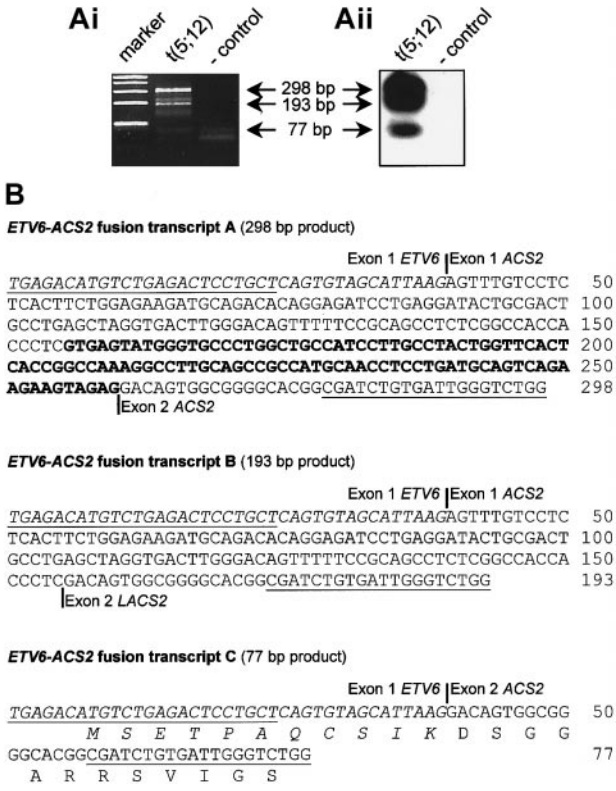

Figure 4. Detection of *ETV6-ACS2* fusion transcripts in the aCML case with a t(5;12)(q31;p13) (case 7). (Ai) RT-PCR confirmed the fusion of *ETV6* to *ACS2,* as identified by 3′-RACE in the t(5;12) case (case 7). (Aii) Hybridization with an internal oligonucleotide revealed the existence of 3 different fusion transcripts, which was confirmed by cloning and sequencing of these RT-PCR products, shown in the lower panel. (B) Representation of the identified fusion transcripts. Open reading frames were determined on the basis of the start codon of *ETV6.* Only transcript C is an in-frame fusion. Exon numbering of *ACS2* is according to Yagasaki et al.[22]

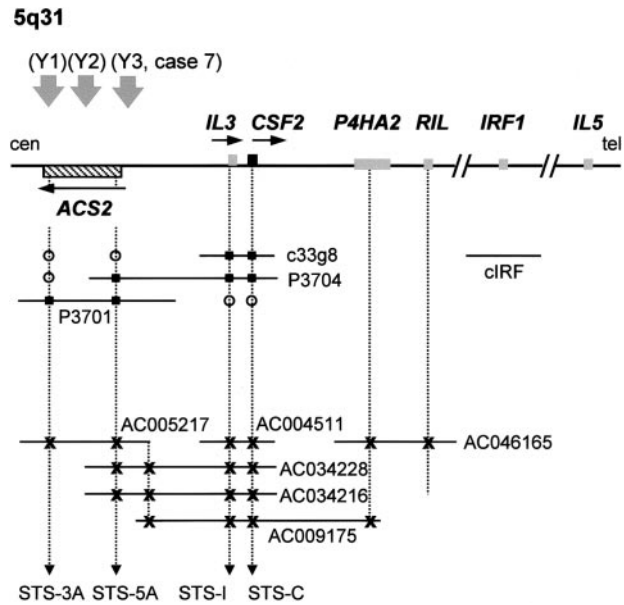

Figure 5. PAC/BAC/cosmid contig covering the *ACS2* and *IL-3/CSF2* loci at 5q31 and localization of the different breakpoints of the t(5;12)(q31;p13). Schematic illustration of the small PAC/cosmid contig linking *ACS2* with *IL-3/CSF2* (not to scale). Database searches identified several partially or completely sequenced genomic clones containing the *ACS2, IL-3, CSF2,* and *P4HA2* genes, confirming the PAC contig. These clones are represented by their accession numbers. Yagasaki et al[22] mapped *IRF1* telomeric to the breakpoints of the t(5;12). A 1 Mb contig was previously described[33] that links *ACS2* to *IL-5* and is in agreement with the map shown here. The 3 t(5;12)(q31;p13) breakpoints described by Yagasaki et al[22] are numbered as Y1, Y2, and Y3. The breakpoint described in this work is indicated with case 7, as described in the text. Genes are represented by boxes, and their transcriptional orientation is indicated by an arrow. The sequenced BAC clones are represented by their accession numbers. tel indicates telomeric side; cen, centromeric side; open circle, negative by hybridization or PCR; filled square, positive by hybridization or PCR; x, positive by electronic PCR/database searches.

reported by Frazer et al,[33] the distance between these genes is most probably 100 kb or less.

Expression of *IL-3* in the t(5;12)(q31;p13)

The expression of *IL-3, CSF2, IL-5,* and *IL-9* was analyzed in a bone marrow sample obtained from the aCML case (case 7). Only expression of *IL-3* could be clearly detected (Figure 6), indicating a good correlation between the position of the breakpoint and the expression of *IL-3* in this myeloid sample. Although *CSF2* is only 10 kb distal to *IL-3,* no expression of this gene was observed. The expression of *IL-5* and *IL-9,* which are not located in the immediate surroundings of the t(5;12) breakpoint, was investigated as a control. In this way a "contamination" of the sample with normal T cells expressing *IL-3* was excluded because expression of *IL-5* and *IL-9* would then also be detected.

Discussion

We report here a further molecular study of the recurrent translocations t(4;12)(q11-q12;p13) and t(5;12)(q31;p13). Our results described in this work together with our previous study[23] and the study of Yagasaki and colleagues[22] clearly indicate heterogeneity in the localization of the breakpoints at both 4q11-q12 (upstream, at the 5′ end or in intron 3 of *CHIC2*) and 5q31 (at the 5′ end, in the middle or at the 3′ end of *ACS2*). In correspondence with this, no common fusion transcript that could explain the pathogenic character of these translocations is generated by either of these translocations. In addition, the *HSG2-ETV6* transcript detected in case 5 with the t(4;12) and the *ETV6-ACS2* transcripts described by Yagasaki et al[22] in cases with a t(5;12) do not represent in-frame fusion transcripts, raising the question as to how these transcripts contribute to the pathogenesis of the respective translocations. Two other AML cases with a t(4;12)(q11-q12;p13) and an *HSG2-ETV6* fusion have been reported,[36] further confirming the heterogeneity of the 4q11-q12 breakpoints.

In fact, the only invariant result of these translocations is the juxtaposition of the chromosomal regions telomeric, respectively, to *CHIC2* on 4q11-q12 and *ACS2* on 5q31 to the *ETV6* locus on

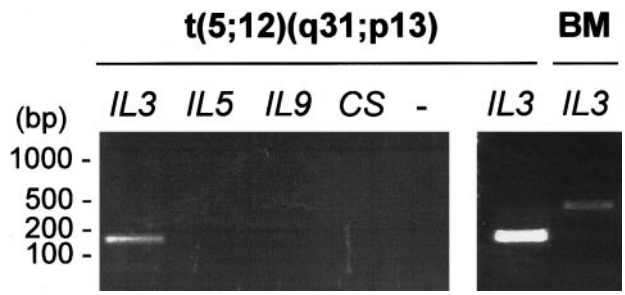

Figure 6. Expression of *IL-3* in the aCML case with a t(5;12)(q31;p13) (case 7). Expression of *IL-3* but not of *CSF2, IL-5,* or *IL-9* was detected in a bone marrow sample from the patient with aCML (case 7). By contrast, high-level expression of *IL-3* could not be detected in control bone marrow cells (BM), indicating ectopic expression of *IL-3* in the t(5;12) case. The faint band observed at 394 bp in control bone marrow is due to a genomic contamination of the RNA sample.

12p13. The juxtaposition of protooncogenes to the *RPN1* locus, the IgH/L loci, or the TCR loci has been well illustrated and results in the deregulated expression of these protooncogenes.[2,3,37] We identified the homeobox gene *GSH2* and the growth factor genes *IL-3* and *CSF2* in the regions adjacent to the breakpoints at, respectively, 4q11-q12 and 5q31 of the t(4;12)(q11-q12;p13) and the t(5;12)(q31;p13). Ectopic expression of *GSH2* was detected in all t(4;12)(q11;p13) cases, with or without the *CHIC2-ETV6* fusion, and expression of *IL-3* was observed in the aCML case with a t(5;12)(q31;p13), also characterized by an *ETV6-ACS2* fusion. These results suggest that ectopic expression of *GSH2* and *IL-3* could be the underlying leukemogenic mechanisms of the t(4;12)(q11-q12;p13) and t(5;12)(q31;p13) cases, independent of the presence of fusion transcripts.

The *Gsh2* gene was previously characterized as a brain-specific homeobox gene important in forebrain and hindbrain formation of the mouse.[38,39] We did not find evidence that *GSH2* is also expressed during hematopoiesis. Interestingly, *GSH2*, belonging to the ParaHox genes,[40] contains a homeobox domain that is very similar to the homeobox of the clustered HOX genes, which are involved in both normal and abnormal hematopoiesis.[41] In addition, overexpression of *GSH2* was found to induce focus formation in the NIH3T3 cell line (Figure 3), illustrating its oncogenic potential.

Evidence for the involvement of *IL-3* in leukemia was described previously by the analysis of the t(5;14)(q31;q32) in B-ALL, involving ectopic expression of *IL-3* as a consequence of its translocation to the *IgH* locus,[35,42] and by analysis of mutations found in the murine WEHI-3B cell line, which illustrated that ectopic expression of *HoxB8* and *IL-3* together results in AML in a mouse model.[43] Although expression of *IL-3* alone produced only a nonneoplastic myeloproliferative disease in this mouse model, it is clear from these studies that ectopic expression of *IL-3*/*IL-3* leads to a proliferative defect and may be involved in the multistep process of leukemogenesis.[43,44] In addition, because *IL-3* is a growth factor regulating eosinophil proliferation and differentation,[45] ectopic expression of *IL-3* could also explain the eosinophilia associated with the t(5;12) cases,[22,25,29-32] as was also observed for the t(5;14)(q31;q32).[35,42] This is supported by the observation that continuous administration of IL-3 leads to a dose- and time-dependent eosinophilia.[46]

Although we clearly detected expression of *GSH2* and *IL-3* in the t(4;12) and t(5;12) cases, several remarks must be taken under consideration when describing this as an abnormal expression. Bone marrow is a heterogeneous mixture of different cell types of which in leukemia only one particular cell was originally mutated and expanded. It is thus possible that the expression of a gene, which is expressed only in a rare bone marrow cell type, would become detectable by RT-PCR in leukemic bone marrow as a consequence of the clonal expansion of this cell type. Second, translocations are known to occur in open chromatin structures, suggesting that the genes found at the breakpoints are transcriptionally active, thus arguing that the detected expression of *GSH2* and *IL-3* in the leukemias might not be abnormal. However, the breakpoints of the t(4;12) and t(5;12) are not very close to *GSH2* or *IL-3* but occur in or at least close to, respectively, the *CHIC2* and *ACS2* genes, which are expressed in the hematopoietic system[22,23] and thus most probably are located in an open chromatine region, providing space for the translocations to occur. The fact that *GSH2* expression could not be detected in any of the analyzed subpopulations highly enriched for $CD34^+$ cells further indicates that *GSH2* is not expressed in the hematopoietic system, although we cannot exclude its expression in a particularly rare bone marrow cell type. However, even if *GSH2* would be expressed in a rare bone marrow cell type that escaped our detection, this would not directly exclude *GSH2* from its involvement in the t(4;12). Transformation might then be the result of the inability to down-regulate *GSH2*, rather than its induced expression. In this regard, the ectopic expression of *Hoxa9* by retroviral insertion in BXH2 mice is a well-characterized example illustrating that the ectopic expression of a gene that is normally expressed in bone marrow progenitor cells can nevertheless be leukemogenic.[47,48]

It has been well established that *IL-3* is expressed by activated T cells, NK cells, and mast cells.[49] Detection of its expression in a CML sample, containing mainly myeloid cells, is suggestive for its abnormal expression in this cell type. If expression of *IL-3* would be the result of the presence of other IL-3–producing cells, then expression of other growth factors such as *CSF2*, *IL-5*, and *IL-9* also would be expected, which was not the case (Figure 6). Although *IL-3* and *CSF2* are located very close to each other, no expression of *CSF2* was detected in the aCML case (Figure 6). Interestingly, this was also observed in B-cell ALL cases with a t(5;14)(q31;q32) involving the IgH locus.[42] Although *IL-3* and *CSF2* have an overlapping expression pattern, *CSF2* expression can also be regulated separately.[49,50] Elucidation of the mechanism by which expression of *IL-3* becomes activated in these leukemias could provide further explanation for this.

The exact mechanism by which expression of *GSH2* and *IL-3* is activated is currently not known. It has become clear that the final transcription level of a gene is determined by the combined action of regulatory sequences that might be either positive or negative and the accessibility of these sequences determined by the chromatin structure and the nuclear architecture.[51,52] Therefore, it might be expected that regulatory sequences as enhancers in *ETV6* and silencers at the partner chromosome can be major contributors, with additional effects from the change in chromatin structure around the breakpoint. Well-known examples involving enhancer-mediated ectopic expression are the translocations involving the *RPN1* locus, the IgH/L loci, or the TCR loci.[2,3,37,53] These studies have also clearly illustrated that the breakpoints can be located up to 200 kb away from the deregulated protooncogene.[3] Other examples of how translocations can influence the expression of genes have been described for the translocations associated with Beckwith-Wiedemann syndrome, which involve aberrant expression of the *IGF2* gene by loss of imprinting,[54,55] and for chromosome rearrangements at the *Kit*/*Pdgfra* loci in mutant mice, affecting the expression of *Kit*.[55,56] In addition to the effects of enhancers and silencers, all translocations are likely to change local chromatin structure and nuclear architecture in its whole, which may be associated with changes in gene expression.

Taken together, our results indicate that the common mechanism of the t(4;12)(q11-q12;p13) cases, with or without the *CHIC2-ETV6* fusion, may be the ectopic expression of *GSH2*. Further analysis of additional cases with breakpoints at the 5′ end of *CHIC2* and analysis of the in vivo leukemogenic properties of *GSH2* will be necessary to fully understand the importance of its expression in the t(4;12)(q11-q12;p13) cases. In addition, our results suggest that the AML-M0 cases represent a subgroup in the t(4;12)(q11-q12;p13) characterized by both the expression of *GSH2* and the *CHIC2-ETV6* fusion. Although expression of *CHIC2-ETV6* does not transform the NIH3T3 cell line, it will be of interest to investigate whether the presence of the *CHIC2-ETV6* fusion plays an additional role in determining the immature phenotype of the AML-M0 cases. With respect to the reported

t(5;12)(q31;p13) cases, both our data and the known leukemia-related properties of *IL-3* are supportive for the ectopic expression of *IL-3* as an important transforming mechanism in these cases. Although we have only been able to investigate a single case, the 3 cases described by Yagasaki et al[22] also involve a translocation of *IL-3* to the der,[12] supporting the possibility of *IL-3*–induced expression by *ETV6* regulatory sequences.

Although many translocations involving *ETV6* have been shown to result in the generation of an in-frame fusion gene, encoding a fusion protein with oncogenic properties, our results indicated the importance of a variant mechanism: the ectopic expression of a protooncogene. Other translocations, such as the reported t(6;12)(q23;p13)[21] and t(7;12)(p15;p13)[57] and the t(10;12)(q24;p13) and t(12;13)(p13;q12) (P. M., unpublished results, 2001), seem not to result in the generation of functionally significant fusion genes. It will be of further interest to investigate whether these cases involve ectopic expression of a protooncogene. Interestingly, several cases of the t(3;12)(q26;p13) have been described, with the 3q26 breakpoints either 5′ or 3′ to *EVI1*, a gene that is ectopically expressed in myeloid malignancies with the inv(3)(q21q26) or the t(3;3)(q21;q26). One of the t(3;12)(q26;p13) cases was cloned and revealed the fusion of *ETV6* to *MDS1*/*EVI1*.[19] However, at least one of the other cases described by Raynaud et al[58] had breakpoints centromeric to *EVI1* and thus could involve ectopic expression of *EVI1* by a similar mechanism as proposed in this work. Identification of candidate protooncogenes for other translocations involving breakpoints at the 5′ end of *ETV6* will be greatly facilitated by the availability of the complete human genome sequence.

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